DEVELOPMENT OF A VACCINE FOR *NEISSERIA MENINGITIDIS* GROUP B BASED ON NATIVE OUTER MEMBRANE VESICLES

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ABSTRACT

A novel approach for development of a meningococcal group B vaccine has been developed. This approach , which uses vesicles or blebs of the meningococcal outer membrane prepared without exposure to detergent or denaturing solvents, allows the outer membrane antigens to be presented to the immune system in their natural conformation and membrane environment. Two strategies for using these native outer membrane vesicles (NOMV) as a vaccine have been investigated. The first is intranasal vaccination using NOMV prepared from a capsule negative mutant, and the second is intramuscular vaccination using NOMV prepared from a double mutant that is capsule negative and expresses a mutant lipopolysaccharide with low endotoxin activity. The intranasal vaccine has been evaluated in mice and rabbits and in two phase 1 clinical studies. The intramuscular vaccine has been evaluated in animals and is currently being evaluated in a clinical study. Results obtained to date with these vaccines are promising and show evidence of a high quality and quite cross reactive antibody response.

1. INTRODUCTION

Meningococcal disease is a threat to military personnel principally during basic training. Large outbreaks or epidemics have historically occurred during major military mobilizations. For example, an epidemic occurred in U. S. Military basic training centers during the Vietnam War in the mid 1960's. Approximately 200-300 cases per year of group B disease were reported among US Army recruits between 1964 and 1966 (Brundage and Zollinger, 1987). The case-fatality rate for US Army personnel during this period was 7.2%. The overall burden of meningococcal disease for the US military between 1964 and 1998 was 3044 cases of which about one-third were group B (Brundage, et al., 2002). Using a case fatality rate of 7.0 % the calculated number of lives lost during that period was 213. The rapid onset of the disease, the importance of early treatment with antibiotics to prevent death, and the known risk to close contacts of cases results in a high level of concern and a high level of disruption of normal operations when an outbreak occurs.

Since the epidemic in the 1960's, group B disease in the U.S. military has remained at a relatively low level with occasional small clusters of cases occurring at different training posts. Group B *N. meningitidis* is currently responsible for about 30 to 70% of all meningococcal disease in North and South America and in most European countries. Over the past 25 years, group B epidemics have occurred in Norway, Cuba, Chile, and Brazil, and New Zealand. Although current incidence in low, the threat of group B meningococcal disease persists. Past experience has demonstrated that use of prophylactic antibiotics to control meningococcal disease among military recruits leads to the emergence of drug resistance.

Five serogroups of meningococci, A, B, C, Y, and W135, are responsible for nearly all cases of systemic meningococcal disease. During the late 1960's and the 1970's effective capsular polysaccharide-based vaccines were developed for four of the five pathogenic serogroups. These polysaccharide vaccines are now being replaced by conjugate vaccines which are T-cell dependent vaccines and more effective in young children. Group B is the sole remaining pathogenic serogroup for which no vaccine is available.

Development of a vaccine for group B disease is a much more difficult challenge than for the other four serogroups. The group B capsular polysaccharide is a poor immunogen due to the fact that it has the same chemical structure ($\alpha 2 \rightarrow 8$ linked polysialic acid) as oligosaccharides present on certain human cells, and therefore may potentially induce autoimmunity. In addition, the major subcapsular (outer membrane) antigens are either antigenically variable, phase variable, or both. The outer membrane protein antigens are closely associated with endotoxin or lipooligosaccharide (LOS) which must be removed or detoxified for the protein antigens to be safe for use as a parenteral vaccine. Also, no good animal model for the disease exists since human beings are the sole host.

We have developed a group B vaccine technology that is based on the use of native outer membrane vesicles (NOMV) or blebs which are released from meningococci during normal growth or can be extracted

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from cells by mild procedures not involving use of detergents or denaturing solvents (figure 1).

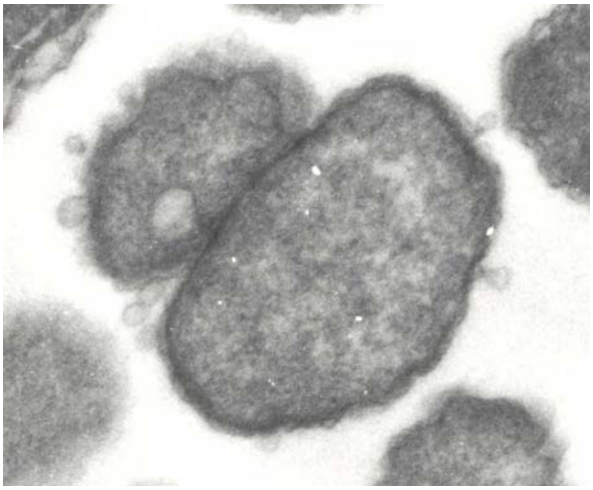

Figure 1. Thin section electron micrograph of *Neisseria meningitidis* cells showing blebbing of the outer membrane. Blebs isolated from pelleted cells using mild procedures constitute native outer membrane vesicles.

We have identified and are evaluating two approaches for safely using NOMV as a vaccine. (1) NOMV prepared from a mutant unencapsulated strain (Δ*synX*) have been safely used in human volunteers as an intranasal vaccine. (2) NOMV prepared from a double mutant strain that is *synX* negative and have genetically detoxified LOS (Δ*lpxL2*) have been safely administered to human volunteers as a parenteral vaccine.

2. METHODS

NOMV vaccines were prepared by extraction without exposure to detergent from cell paste as described by Drabick, et al. 2000.

Serum bactericidal antibodies were measured using human serum as a source of complement as described by Moran, et al. 1994. Total antigen specific antibody was measured by a quantitative enzyme linked immunosorbant assay as described by Saunders, et al. 1997. Native outer membrane vesicles or purified LOS non-covalently complexed to an equal weight of bovine serum albumin was used as antigen.

The general safety test and the rabbit pyrogen test were conducted according to regulatory requirements under current good laboratory practice by contract to BioReliance, Inc. The TNF-release assay was performed using fresh human monocytes as describe by Fisseha, et al. 2005.)

3. RESULTS

3.1 Intranasal Vaccine Based on NOMV

NOMV vaccine was prepared from strain 9162 (B:15:P1.7-2,3:L3,7) Δ*synX,* which is unable to synthesize sialic acid and is therefore capsule negative and expresses unsialylated LOS. Its composition was consistent with that of intact outer membrane, containing approximately 20-25μg of LOS per 100 μg of protein (figure 2). Major outer membrane proteins present in the NOMV, and identified by binding of specific monoclonal antibodies on western blots, included PorB, PorA, RmpM, Opa, TbpB, Lip, and FetA. In addition, many minor outer membrane proteins were present. Since the cells were not exposed to detergent or organic solvents, the phospholipid bilayer remained intact. The yield of NOMV was 2-3 mg of NOMV protein per gram of cell paste. The NOMV was examined by negative stain electron microscopy to determine the size and purity of the vesicles. The result (figure 2) showed well-defined vesicles ranging in size from 0.5 to 2.0 μ in diameter.

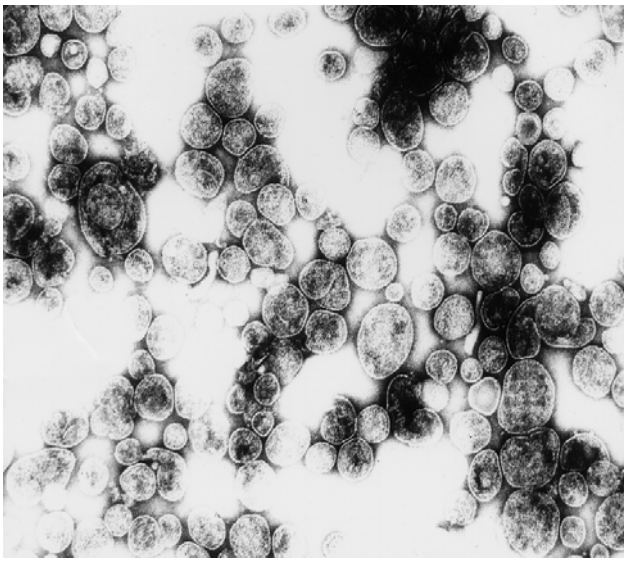

Figure 2. Negative stain electron micrograph of NOMV prepared from meningococcal group B strain 9162 Δ*synX* and used as a intranasal vaccine.

Studies of the effectiveness and safety of NOMV as an intranasal vaccine were carried out in mice and rabbits (Saunders, et al. 1999; Shoemaker et al. 2005). The NOMV vaccine was found to induce a strong bactericidal antibody response that was similar in magnitude to that induced by parenteral vaccination. In addition, a significant muscosal antibody response was demonstrated. One distinguishing characteristic of the antibody response to intranasal vaccination with NOMV

as compared to parenteral vaccination with detergent extracted vesicles was the more prominent response to the LOS component of the NOMV and a higher degree of cross reactivity with heterologous strains. The response of rabbits to the LOS component of the NOMV following intranasal vaccination is shown in figure 3. Of interest is the specificity of the antibodies. Stronger binding was observed to the L8 LOS, which has a truncated oligosaccharide structure, than to the L3,7 LOS which was present in the vaccine and has full length oligosaccharide containing the lacto-N-neotetraose group. Lacto-N-neotetraose is a precursor to some of the blood group antigens. The stronger binding to L8 suggests the antibody response is mostly directed against the core region of the LOS rather than the lacto-N-neotetraose containing alpha chain.

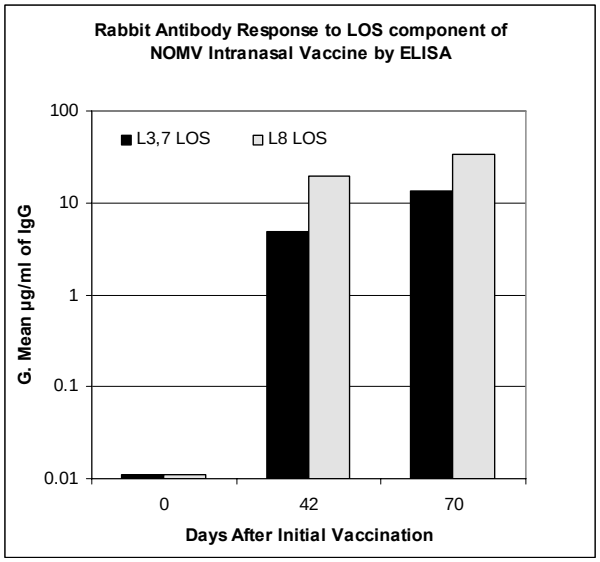

Figure 3. The anti-LOS antibody response of rabbits to intranasal vaccination with NOMV vaccine measured by quantitative ELISA is shown. Rabbits were given 100 µg of vaccine intranasally at 0, 28 and 56 days.

In human phase 1 clinical studies with NOMV vaccine given intranasally (Drabick et al. 2000 ; Katial et al. 2002), the vaccine was tolerated very well in doses up to 400 µg in spite of the considerable amount of LOS (endotoxin) in the NOMV vaccine (82 µg/400 µg dose on NOMV). Overall, the immune response in humans was weaker than in mice or rabbits, but the antibodies induced appeared to be very effective in terms of bactericidal activity. In the best group, 70% of the volunteers seroconverted (4-fold or greater increase in serum bactericidal titer). Figure 4 shows the bactericidal antibody response of group 4 volunteers (400 µg dose given 3 times at four-week intervals). While the titers were not very high, the increases were significant and the antibodies persisted. A mucosal antibody response was also induced by the vaccine as evidenced by a significant increase of specific IgA and IgG antibodies in nasal washes of most volunteers.

The specificity of the bactericidal antibodies induced in human volunteers by NOMV vaccine given intranasally was analyzed. The bactericidal antibodies were not predominantly subtype (PorA) specific. In several of the sera tested 50% to 70% of the bactericidal activity was removed by purified LOS.

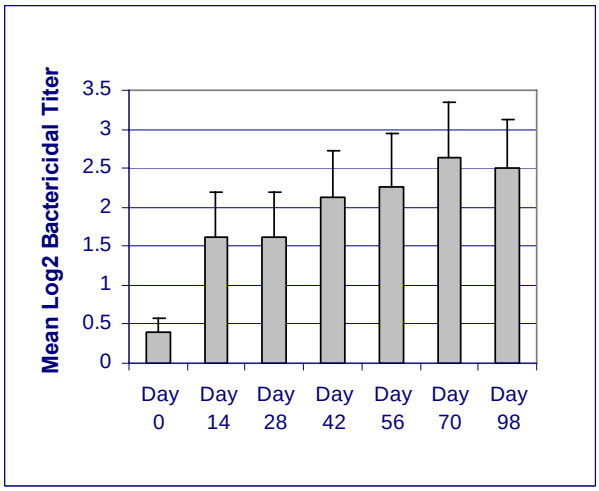

Figure 4. Bactericidal antibody response of volunteers from one group of 8 volunteers who received NOMV vaccine, lot # 0123, intranasally. The test strain was the homologous strain, 9162 (B:15:P1.7-2,16: L3,7).

Overall, intranasal vaccination with NOMV vaccine showed promise, but the antibody responses were not as robust as seen in animals or as desirable for a successful group B vaccine. The addition of a safe and effective mucosal adjuvant may be required for this approach to be successful. This approach deserves further evaluation, but we desired first to compare the intranasal approach with an alternative approach for using NOMV as a vaccine, which is the use of NOMV prepared from a strain with genetically detoxified LOS delivered intramuscularly.

3.2 Parenteral Vaccine Based on NOMV

Use of NOMV as prepared for intranasal vaccination was not suitable for use as a parenteral vaccine because of the high level of endotoxin in the outer membrane (about 20-25% relative to protein). Thus in order to reduce the toxicity of the NOMV an additional mutation was put in the vaccine strain that modified the structure of the lipid A portion of the LOS, which is responsible for LOS toxicity. One of two genes, *lpxL1* or *lpxL2*, was disabled by insertional inactivation. These two genes

code for acyl transferases which attach secondary, acyl-oxy-acyl linked fatty acids to the lipid A, inactivation of *lpxL1* results in penta-acyl Lipid with one of two branching fatty acids missing, and inactivation of *lpxL2* results in a tetraacyl lipid with both the secondary fatty acids missing.

The effect of these mutations on the vaccine strains and the toxicity and immunogenicity of the NOMV was investigated (Fisseha et al. 2005). In summary, the *lpxL2* mutation rendered the vaccine strain quite sickly, with slower growth in liquid culture, greater sensitivity to surfactants, reduced viability in stationary phase of growth, and a significantly lower amount of LOS in the outer membrane (about 3-5% as compared to 20-25% without the mutation). The *lpxL1* mutation, on the other hand had minimal impact on the growth of the strain and the LOS content of the NOMV. The relative toxicity of NOMV prepared from the two mutants was compared in several assay systems. The results given in table 1 show that endotoxin activity as measured by the Limulus Amoebocyte lysate (LAL) assay was essentially the same for the two mutant NOMVs as for NOMV from a strain with wild type LOS.

Table 1. Comparison of three methods for determination of endotoxin activity associated with NOMV vaccines.

Mutations in NOMV Vaccine Strain	**LAL Assay***	**Rabbit Pyrogen Test#**	**TNF-α Release by Human Monocytes****
Δ*synX* Δ*lpxL2*	0.9 X 10^6	2.0 µg/kg	3 ng
Δ*synX* Δ*lpxL1*	2.4 X 10^6	0.05 µg/kg	1.5 ng
Δ*synX*	2.5 X 10^6	0.01 µg/kg	0.02 ng

*Endotoxin units
#Highest concentration of vaccine that was nonpyrogenic
**Concentration of NOMV vaccine required to induce half the maximum release of TNF-α from human monocytes.

When evaluated by the rabbit pyrogen test, the *lpxL1* mutant NOMV was about 5-fold less toxic than NOMV with wild type LOS and the *lpxL2* mutant NOMV was 200-fold less active. Somewhat different results were obtained when the release of TNF-α from human monocytes was used as a measure of endotoxin activity. In the TNF release assay the *lpxL1* was only slightly more active than the *lpxL2* mutant NOMV, and both were approximately 100-fold less active than NOMV from a strain with wild type LOS (Table 1). These data suggested that the *LpxL2* mutant NOMV would likely be safe for parenteral injection into humans, but the *LpxL1* mutant LOS may or may not be safe for parenteral use, depending on whether the rabbit pyrogen or the TNF release assay was the more accurate predictor of the vaccine's behavior in humans.

The immunogenicity of the NOMV prepared from *lpxL1* or *lpxL2* mutant strains was studied in mice and rabbits. NOMV vaccine from a double mutant strain with knockouts of the *lpxL1* gene and the *synX* gene was tested in a dose response study in mice. Mice were vaccinated intraperitoneally at 0, 4 and 8 weeks, and blood was taken at 0, 7 and 10 weeks. Results of bactericidal assays on the mouse sera are shown in figure 5. A clear dose-response over the range of doses tested was observed and the geometric mean levels antibody induced were quite good, though lower than those obtained with wild type NOMV or *lpxL1* NOMV. Several different adjuvants were tested and, it was found that immunogenicity could be restored to wild type levels by use of an adjuvant. Apparently, the *lpxL2* mutation, which detoxifies the LOS also removes the inherent adjuvant activity of the LOS. In addition, the much reduced LOS content of the *lpxL2* NOMV essentially removed the benefit of the LOS as an antigen. However, in this vaccine the outer membrane proteins are presented to the immune system in a native conformation and membrane environment, and the toxicity was very low compared to wild type (GM log2 titer of 8.5 ± 0.4 for 1 µg dose).

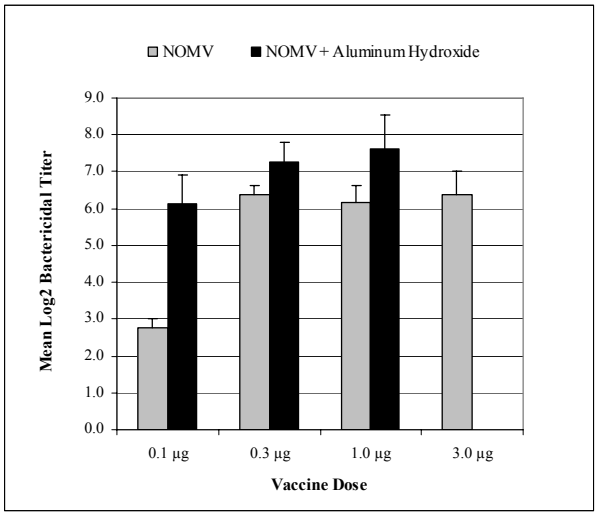

Figure 5. Bactericidal antibody response of mice to different doses of NOMV vaccine prepared from a double mutant strain with knockouts of the s*ynX* and *lpxL2* genes.

The stability of the vaccine was evaluated by storing the vaccine at 4º C, 22º C, or 37º C for 1, 3 or 6 months and testing the vaccine for evidence of breakdown by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) with Coomassie Blue staining and by immunization of mice and measurement of the resulting bactericidal antibody response. The results of the SDS-

PAGE analysis showed some gradual breakdown of the vaccine over time as evidenced by some broadening and fading of the protein bands on the gel, which increased with increasing time and temperature of storage. There was little if any change with storage at 4° C for up to 6 months, but storage at 37°C for over 1 month resulted in pronounced broadening of the protein bands. When the vaccine samples were tested for their ability to induce bactericidal antibodies in mice, there was no loss in potency except for the sample held at 37° C for 6 months, which gave a lower titer than the control sample. Thus, the NOMV appear to be very stable. Consistent with these observations was the finding that the NOMV prepared for intranasal vaccination in earlier experiments were stored for up to 4 years at 4° C without substantial loss in immunogenicity.

A clinical lot of NOMV vaccine (Lot # 1119) was prepared from strain 44/76 Δ*synX* Δ*lpxL2* to test the safety and immunogenicity of the vaccine in human subjects as a proof of principle for moving ahead with this vaccine approach. The clinical lot of vaccine was tested for sterility, identity, composition, and potency in both mice and rabbits. In addition, the vaccine was analyzed in the general safety test, the rabbit pyrogen test, and for the induction of human monocytes to release TNF-α (endotoxin activity). The vaccine passed the rabbit pyrogen test at a level of 2 µg/kg. It passed the

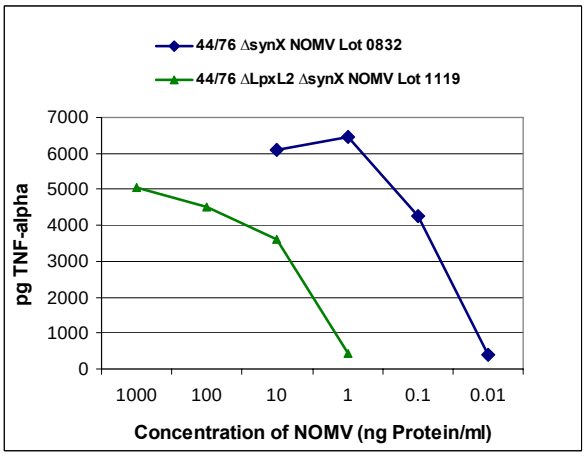

Figure 6. Induction of TNF-α release from human monocytes by NOMV vaccine lot 1119 and lot 0832 with wild type LOS.

general safety test and when tested in the TNF- α release assay using human monocytes, it was shown to be about 100-fold less Endotoxin activity than NOMV from a strain with wild type LOS (figure 6).

Potency tests in mice and rabbits were encouraging and demonstrated that the vesicles retained good immunogenicity even though most of the adjuvant activity normally associated with LOS had been lost due to the *lpxL2* knockout and the low level of expression of LOS in the mutant strain. The geometric mean bactericidal antibody titers in rabbits were about 2 fold lower than those observed for NOMV with wild type LOS. The results of the rabbit immunizations are shown in figure 7. Three formulations were used: 25 µg without adjuvant, 25 µg with aluminum hydroxide (Rehydragel LV, Reheis, Inc.) and 50 µg without adjuvant. In rabbits there was no significant difference in bactericidal antibody response for the different formulations. The geometric mean titers induced by vaccine lot 1119 were 2 to 3-fold lower than those induced by control wild type NOMV.

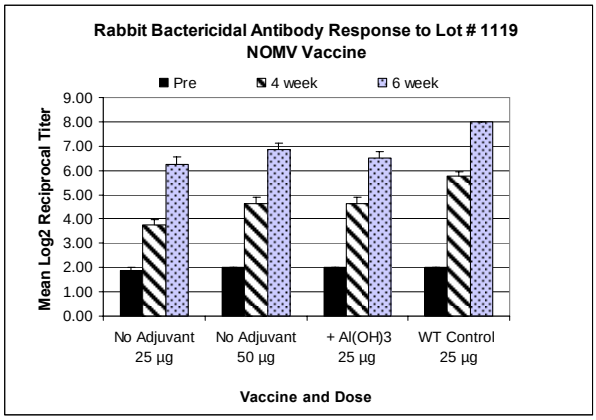

Figure 7. Bactericidal antibody response of rabbits to NOMV vaccine lot 1119 prepared for clinical use. Rabbits were vaccinated with the indicated dose and formulation at 0 and 4 weeks. The bactericidal test strain was the wild type parent of the vaccine strain, 44/76.

A dose-response study was conducted in mice over the range of 0.1 to 3.0 µg. Three doses of vaccine were given intraperitoneally to the mice at 0, 4 and 8 weeks. The bactericidal antibody responses were measured using the wild type strain 44/76 from which the vaccine strain was derived. The results of the mouse potency study are shown in figure 8. The results of the rabbit and mouse immunogenicity studies along with the safety studies were encouraging and led to a decision to test the vaccine in a phase 1 clinical study. This study is currently in progress.

4. CONCLUSIONS

Results to date indicate that a vaccine based on NOMV has a number of advantages over certain competing technologies such as deoxycholate-extracted outer membrane vesicles, which is the most extensively studied meningococcal group B vaccine approach. The NOMV are easy to prepare; induce an immune response

similar to that induced by natural infection (not strongly subtype specific); maintain key antigens in their native

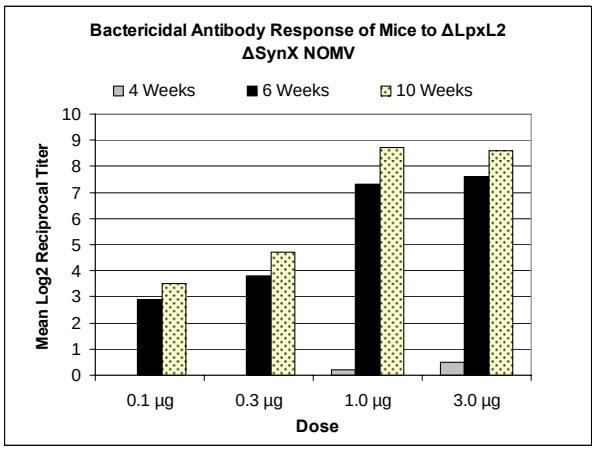

Figure 8. Bactericidal antibody response of mice to different doses of NOMV vaccine lot 1119.

conformation and environment, which results in the induction of antibodies that can better recognize the intact organism; and include LOS, which is an important antigen that can induce a long-lasting bactericidal antibody response with relatively broad specificity. With the completion of the phase 1 clinical study currently in progress, we will have completed the evaluation of two different approaches for using native outer membrane vesicles as a vaccine. When the clinical study is completed, we will down select for the best of the two technologies and carry forward that approach to the further development of a multivalent vaccine for group B that is capable of protecting military personnel against group B disease caused by all of group B strains.

The extension of this technology to overcome the problem of antigenic variation among group B strains will be addressed by preparation of a multivalent NOMV vaccine prepared from three antigenically diverse strains that have been genetically modified to increase expression of desirable antigens and block expression of undesirable antigens. These strains have been identified and most of the desired genetic modifications have been completed. The final version of this vaccine will need to be combined with the existing tetravalent A, C, Y, W135 vaccine to provide protection to military personnel against all pathogenic serogroups of meningococci.

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The research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*. NRC Publication. 1996 edition. Studies involving human subjects were conducted according to current good clinical practice guidelines and with approval and continuing review by the WRAIR Human Use Research Committee and the Human Subjects Research Review Board of the U. S. Army Medical Research and Materiel Command. Vaccines were tested under an approved FDA Investigational New Drug Application.

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